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Sequence Note

Transmitted Antiretroviral Drug Resistance Surveillance among Newly HIV Type 1-Diagnosed Women Attending an Antenatal Clinic in Entebbe, Uganda

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Abstract

To evaluate transmitted HIV-1 drug resistance and study the natural polymorphism in *pol* of HIV-1 strains of newly diagnosed women attending an antenatal clinic in Uganda we sequenced the protease and reverse transcriptase genes for 46 HIV-1 strains from the threshold surveillance. Of the 46 sequences analyzed, 48.0% were subtype A1 ($n = 22$), 39.0% subtype D ($n = 18$), 2.0% subtype A2 ($n = 1$), 2.0% subtype C ($n = 1$), and 9.0% intersubtype recombinant A1/D ($n = 4$). Overall, many minor mutations were identified in the protease sequences. None of the strains had major associated mutations to any RTI drug or drug class interest after genotyping 37 samples of our cohort. The HIV drug resistance prevalence estimate in Entebbe following the HIVDR-TS methodology is less than 5% as set out by WHO guidelines.

Introduction

TREATMENT ACCESS PROGRAMS such as the World Health Organization's 3 by 5 plan to treat 3 million people by the end of 2005 (3 by 5 progress report, June 2005, WHO/UNAIDS) and the President's Emergency Plan for AIDS Relief (PEPfAR) have promoted significant access to antiretroviral therapy (ART) in low- and middle-income countries. As of June 2005, about 500,000 people were receiving ART in sub-Saharan Africa, although the regional coverage rate was still 11% of the estimated patients with CD4 cell counts less than 300/mm^3 (2% of all HIV-infected patients in this region).[1] Approximately 6.4% of adults in Uganda are HIV-1 infected. In Uganda antiretroviral programs began in 1992 and by the end of June 2006 over 110,000 patients were on ART, provided through about 300 facilities.[2]

The potential barriers to long-term success (such as intermittent drug supply, drug stock-outs, poor patient monitoring, incorrect prescribing practices, and low adherence) as well as the need to begin programs quickly to treat millions of individuals have raised fears that the aggressive plan to roll out ART, particularly in Africa, may generate an epidemic of drug-resistant strains of HIV.[3] This was illustrated in certain African countries, where suboptimal treatment together with inappropriate clinical and laboratory follow-up led rapidly to a high level of drug resistance.[4,5] Data from industrialized countries suggest that the transmission of drug-resistant HIV is an emerging public health problem. The prevalence of resistance mutations in newly infected individuals ranges between 10% and 25% in Europe and the United States.[6,7] In resource-limited countries a large number of epidemiological studies have addressed the important issue of transmission of drug-resistant HIV. Unfortunately, the results of different studies are difficult to compare because of substantial dissimilarities in assay methodology, definitions used to classify drug resistance, the time period in which the data were collected, and the populations under study. There are very few studies in sub-Saharan Africa on transmission of resistant viruses in newly diagnosed or drug-naive individuals and these studies report a prevalence that ranges between 2 % and 10%.[8–11] A minimum-resource method called the HIV drug resistance threshold survey (HIVDR-TS) has been developed for use in resource-limited countries, targeting geographic settings where ART is al-

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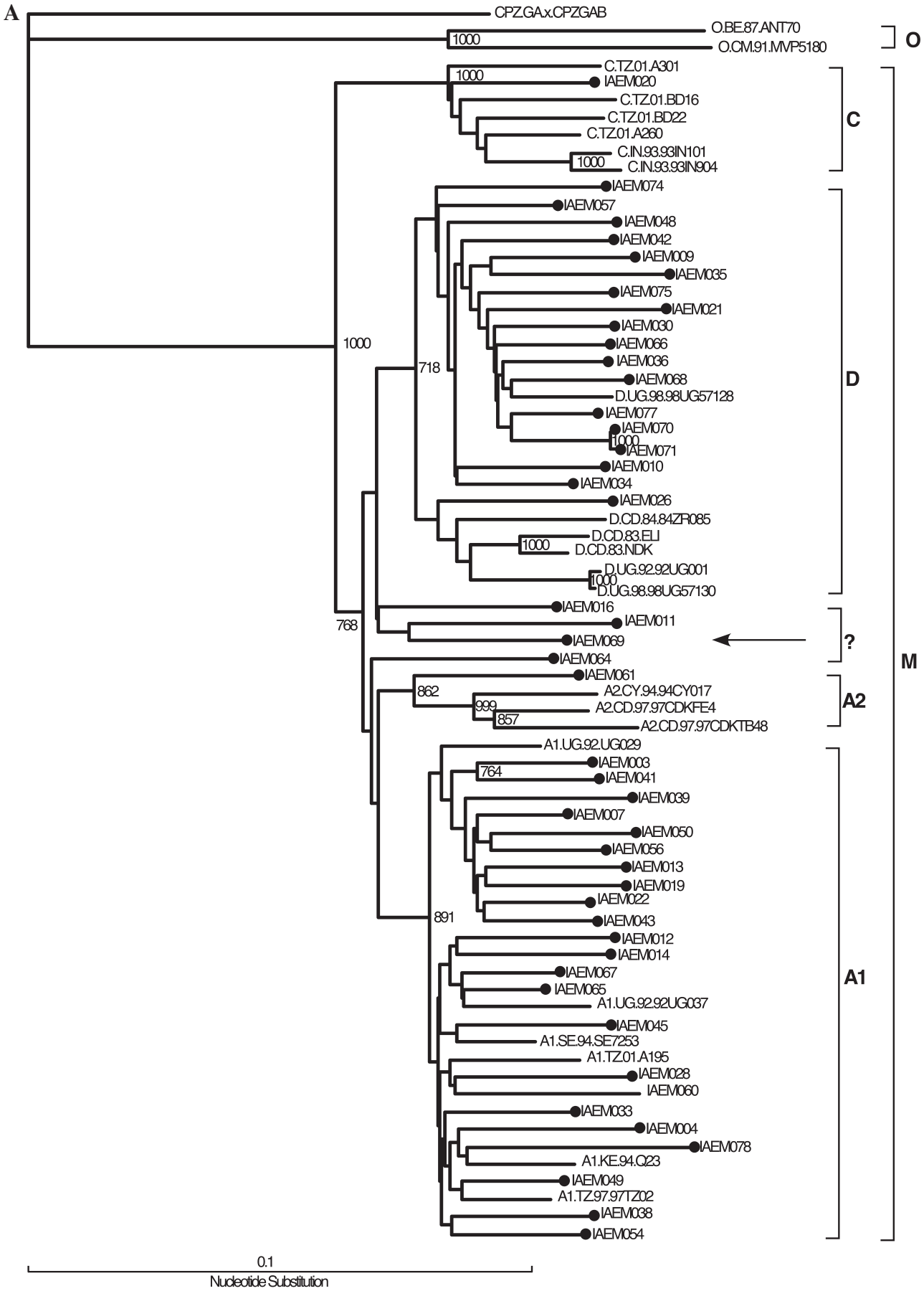

FIG. 1.

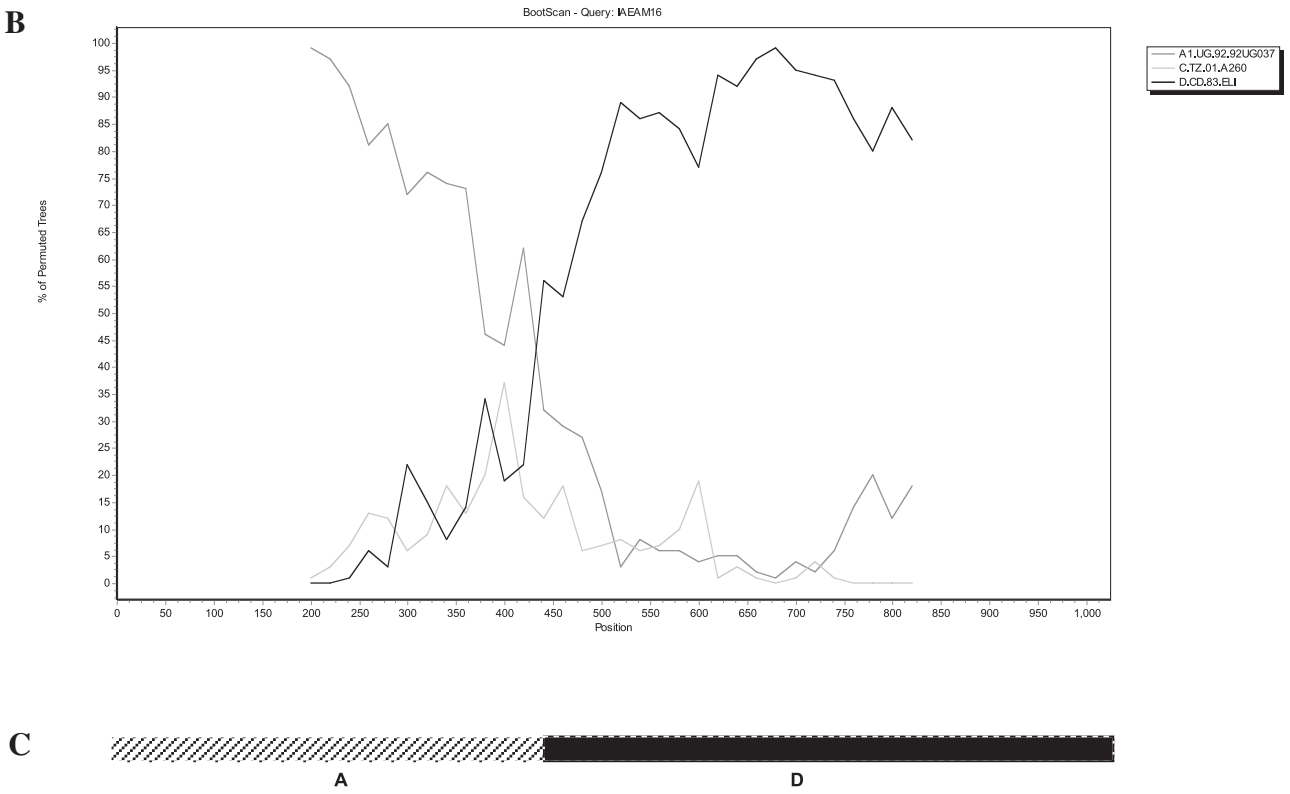

FIG. 1. (**A**) Phylogenetic tree of HIV-1 PR/RT sequences from 46 HIV-1 group M isolates from Entebbe and 23 reference sequences retrieved from the Los Alamos database. The bootstrap value at each node represents the number among 1000 bootstrap replicates that supports the branching order. Bootstrap resampling of 65% or higher is shown. Brackets on the right represent the major group M subtypes. Newly derived sequences are marked with a filled circle (●) and novel unique recombinant form A1/D is shown by an arrow. GenBank accession numbers for the reference sequences are A1.KE.93.Q23–17-accession number (ACC) AF004885, A1.TZ.01.A195 ACC AY253306, A1.TZ.97.97TZ02 ACC AF361872, A1.UG.92.92UG029 ACC U08767, A1.UG.92.92UG037 ACC U51190, A1.SE.94.SE7253 ACC U76165, A2.CY.94.94CY017 ACC AF286237, A2.CD.97.97CDKFE4 ACC AF286240, A2.CD.97.97CDKTB48 ACC AF286238, C.IN.93.93IN101 ACC AB023804, C.IN.93.93IN904 ACC AF067157, C.TZ.01.BD16 ACC AY253322, C.TZ.01.A260 ACC AY253310, C.TZ.01.A301 ACC AY253312, D.CD.83.ELI ACC K03454, D.CD.83.NDK ACC M27323, DCD.84.84ZR085 ACC U88822, D.CD.92.92UG001 ACC AJ320484, D.CD.98.98UG57128 ACC AF484502, D.CD.98.98UG57130 ACC AF484504, O.CM.-.ANT70 ACC L20587, O.CM.91.MVP5180 ACC L20571, and CPZ.GA.-.CPZGAB ACC X52154. (**B, C**) SimPlot analysis was used to map precisely the recombination breakpoint of Ugandan unclassifiable PR/RT (approximately 1030 nt) sequences IAEAM16 (showing the recombination between subtypes A1 and D). The bootscanning analysis was performed against reference strains from clades A (strain A1.UG.92.92UG037), C (C.TZ.01.A260), and D (D.CD.83ELI). (**C**) Segments derived from subtypes A and D are shown.

ready in use or is being rapidly scaled up. Well-functioning ART programs should result in HIV transmitted drug resistance remaining in the category of <5% to each drug in the first line therapy.[12]

In this study we evaluated transmitted HIV drug resistance in a cohort of newly HIV-1-diagnosed, young pregnant women, in which it is likely that the infection has occurred only very recently. Currently, this information is lacking in Uganda and hence the need to monitor and document the prevalence of resistant viruses in Kampala and Entebbe where treatment has gone on longest in the country and use these data to inform drug access initiatives.

Materials and Methods

A binomial sequential sampling method for classification of antiretroviral drug resistance into prevalence classes in prime gravid adolescents and young pregnant females aged 13–22 years with no previous positive HIV test, no exposure to antiretroviral drugs, and CD4 count ≥500 cells/ml was carried out in a semiurban setting at Entebbe Grade B Hospital in Uganda. After obtaining ethical clearance and informed consent, blood specimens were drawn from newly diagnosed HIV-1 women during 2006 and 2007. A total of 889 pregnant women were tested for HIV-1 infection; 61 were found positive (6.8%), 22 specimens were excluded because their CD4 count was below 500 cells/ml, and 37 qualified for inclusion in the WHO drug resistance threshold surveillance protocol. Sequential sampling methods are best used in situations in which classification (e.g., into prevalence classes) of a population or community is useful and where the emphasis is on decision making (whether or not to intervene) with a sample size of 34–47 consecutive antenatal clinic (ANC) attendants.

HIV-1 RNA was extracted from 200 μl of blood plasma using the Qiamp viral RNA mini kit (Qiagen Inc, Chatsworth, CA). Polymerase gene-specific primers were used for reverse transcriptase, followed by nested polymerase chain reactions (PCRs) to amplify a 1030-base pair *pol* gene encompassing amino acids 1–99 of protease (PR) and 1–242 of reverse transcriptase (RT). The PCR products were then purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced in the sense and antisense direction with a set of nested primers.[9] All sequencing reactions were performed using an in-house assay already optimized/validated at MRC/UVRI using a Beckman CEQ 8000 automated capil-

Table 1. Epidemiologic and Genetic Information for Newly Diagnosed HIV-1-Infected Antenatal Women Studied in Entebbe, Uganda

			Genetic subtype[a]				*Drug resistance-associated mutations*				
Sample ID	*Age (years)*	*CD4 count (cells/μl)*	*Accession number(s)*	*Pol-PR*	*Pol-RT*	*URFs*	*1°*	*PR*	*2°*	*1°RT*	*2°*
IAEM003	21	549	EU306745	A1	A1	—	—	*I13V, M36I, L63P, H69K*		—	—
IAEM004	20	740	EU306746	A1	A1	—	—	*I13V, M36I, L63P, H69K*		—	—
IAEM007	18	819	EU306747	A1	A1	—	—	*I13V, M36I, L63P, H69K*		—	—
IAEM009	18	427[b]	EU306748	D	D	—	—	*L10V, 113V, M361*		—	—
IAEM010	18	549	EU306749	D	D	—	—	*I13V, M36I, L63P*		—	—
IAEM011	17	805	EU306750	A1	D	A1/D[c]	—	*I13V, M36I, L63P*		—	—
IAEM012	17	654	EU306751	A1	A1	—	—	*L10V, I13V, K20R, M36I, H69K*		—	—
IAEM013	20	570	EU306752	A1	A1	—	—	*I13V, M36I, H69K*		—	—
IAEM014	19	570	EU306753	A1	A1	—	—	*L10V, I13V, M36I, H69K*		—	—
IAEM016	18	682	EU306754	A1	D	A1/D[c]	—	*I13V, M36I, H69K*		—	—
IAEM019	19	814	EU306756	A1	A1	—	—	*I13V, K20R, M36I*		—	—
IAEM020	21	738	EU306757	C	C	—	—	*M36I, H69K*		—	—
IAEM021	18	999	EU306758	D	D	—	—	*I13V, L63P*		—	—
IAEM022	21	365[b]	EU306759	A1	A1	—	—	*I13V, M36I, H69K*		—	—
IAEM026	22	578	EU306760	D	D	—	—	*I13V, L63P*		—	—
IAEM028	19	970	EU306761	A1	A1	—	—	*M36I, H69K*		—	—
IAEM030	19	486[b]	EU306762	D	D	—	—	*I13V, K20I, M36I, L63P*		—	—
IAEM033	19	1247	EU306764	A1	A1	—	—	*I13V, M36I, L63P, H69K*		—	—
IAEM034	17	998	EU306765	D	D	—	—	*L10V*		—	—
IAEM035	18	584	EU306766	D	D	—	—	*I13V, K20R, M36I*		—	—
IAEM036	20	486	EU306767	D	D	—	—	*I13V, M36I*		—	—
IAEM038	19	761	EU306768	A1	A1	—	—	*I13V, M36I, H69K*		—	—
IAEM039	18	556	EU306769	A1	A1	—	—	*I13V, M36I, L63P, H69K*		—	—
IAEM041	18	575	EU306770	A1	A1	—	—	*I13V, M36I, H69K*		—	—
IAEM042	20	627	EU306771	D	D	—	—	*I13V, V77I*		—	—
IAEM043	20	697	EU306772	A1	A1	—	—	*I13V, M36I, H69K*		—	—
IAEM045	20	501	EU306773	A1	A1	—	—	*L10V, I13V, M36I, H69K*		—	—
IAEM048	16	452[b]	EU306775	D	D	—	—	*I13V, M36I*		—	—
IAEM049	18	709	EU306776	A1	A1	—	—	*I13V, M36I, H69K*		—	—
IAEM050	21	551	EU306777	A1	A1	—	—	*I13V, M36I, L63P, H69K*		—	—
IAEM054	21	505	EU306778	A1	A1	—	—	*I13V, M36I, L63P, H69K*		—	—
IAEM056	na[d]	574	EU306779	A1	A1	—	—	*I13V, M36I, L63P, H69K*		—	—
IAEM057	19	561	EU306780	D	D	—	—	*I13V, L63P*		—	—
IAEM060	21	457[b]	EU306781	A1	A1	—	—	*I13V, M36I, H69K*		—	—
IAEM061	17	638	EU306782	A1/A2	D	A1/A2/D[c]	—	*L10V, M36I*		—	—
IAEM064	20	457[b]	EU306783	A1	D	A1/D[c]	—	*M36I*		—	—
IAEM066	19	509	EU306785	D	D	—	—	*M36I, K20R, A71V*		—	—
IAEM067	19	400[b]	EU306786	A1	A1	—	—	*M36I*		—	—
IAEM068	18	676	EU306787	D	D	—	—	*K20R, M36I*		—	—
IAEM069	18	680	EU306788	A1	D	A1/D[c]	—	*M36I, L63P*		—	—
IAEM070	19	557	EU306789	D	D	—	—	*I13V, M36I*		—	—
IAEM071	21	600	EU306790	D	D	—	—	*M36I*		—	—
IAEM074	23	683	EU306791	D	D	—	—	*I13V, K20R*		—	—
IAEM075	21	647	EU306792	D	D	—	—	*M36I*		—	—
IAEM077	19	499[b]	EU306793	D	D	—	—	*M36I*		—	—
IAEM078	21	726	EU306794	A1	A1	—	—	*I13V, K20I, M36I, H69K*		—	—

[a]Genotyping of human immunodeficiency virus type 1 (HIV-1) *pol* fragments (1030 bp) encompassing *pol–protease (Pol-PR)* and *pol–reverse transcriptase (Pol-RT)*.

[b]Specimens with CD4 cell count <500 are not eligible for HIVDR-TS.

[c]Possible recombination between subtype A1 and D within the region. *1°*, primary mutation of drug resistance; *2°*, secondary mutation of drug resistance; amino acid change without parentheses denotes International AIDS Society (IAS 2007).[15]

[d]na, not available.

lary DNA sequencer. The chromatogram files were read using the Sequencher 4.7 program (GeneCodes, USA) and edited with the BioEdit program v5.0.9 (Hall, 1999).

Phylogenetic analysis and subtyping

Neighbor-joining phylogenetic trees including reference *pol* sequences ($n = 25$) were constructed using Clustal W and then drawn using Tree view PPC version 1.6.6 (Institute of Biochemical and Life Sciences, Scotland, UK). To ensure the quality of the data set, each submitted sequence was checked before inclusion using a sequence quality assessment tool (SQUAT). Sequences that contained stop codons and individual resistance codons with ambiguities consisting of more than two bases per nucleotide position or of more than two ambiguities per codon were excluded from the analysis. Bootstrap resampling (1000 data sets) of multiple alignments was performed to test the statistical robustness of the trees. Recombination was assessed using SimPlot v3.5.1 (100 bootstrap replicates, 400-bp sliding window, and 50-bp step). The Kimura-2 parameter was calculated with the DNADIST program in the PHYLIP package.[13,14]The subtypes were determined for all specimens with the CD4 cell counts >350, while the ART DR was determined in the 37 that fulfilled protocol criteria.

Genotypic resistance analysis

Genotypic resistance was defined as the presence of one or more resistance-related mutations as specified by the consensus mutation figures of the International AIDS Society-USA (IAS-USA).[15] The emergence of amino acid substitutions associated with resistance to RTIs and PIs has been extensively characterized, and these substitutions can be classified into major (primary) and accessory/minor (secondary) mutations. Major mutations lead to a several-fold decrease in sensitivity to one or more ARTs. Accessory mutations may not result in a significant decrease in sensitivity but are associated with an increase in viral fitness (replication capacity).[16]

Based on subtype B consensus sequences, mutations leading to resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors (NRTI and NNRTI) are well defined and differ between the two classes of inhibitors. The most common major RT mutations leading to NRTI resistance occur at positions 41, 62, 65, 67, 69, 70, 74, 75, 77, 115, 116, 151, 184, 210, 215, and 219 (16 in total), and major mutations leading to NNRTI resistance are known to occur at positions 100, 103, 106, 108, 181, 188, 190, 225,[17] and 236 (9 in total).

Based on subtype B sequences, drug-resistance mutations in the protease region at positions 10, 13, 16, 20, 24, 30, 32, 33, 34, 36, 43, 46, 47, 48, 50, 53, 54, 58, 60, 62, 63, 64, 71, 73, 76, 77, 82, 84, 85, 88, 89, 90, and 93, 33 in total, have been shown to be associated with resistance to protease inhibitors (PI).[17]

Accession numbers

The DNA sequence of HIV-1 *pol* –PR/RT determined as part of this study has been submitted to GenBank under the following accession numbers: EU306745–EU306794.

Results

HIV-1 subtype distribution

Of the 46 specimens from 46 pregnant women with CD4 cell counts >350 analyzed, 48.0% were subtype A1 ($n = 22$), 39.0% subtype D ($n = 18$), 2.0% subtype A2 ($n = 1$), 2.0% subtype C ($n = 1$), and 9.0% intersubtype recombinant A1/D ($n = <1$) with a recombination breakpoint confirmed for sample IAEM016 between 510 and 515 bp based on bootscanning analysis (Fig. 1A and B).

Sociodemographic variables

Of these 46 patients only 37 were qualified according to the WHO criteria, a median CD4 count of 650 cells/mm^3. The mean age of the 37 patients was 19.2 ± 1.5 (SD) years and over 50% of mothers were below 20 years old (Table 1). The mean age at first sexual encounter was 15.9 years. None of the mothers reported having had a miscarriage at the time of the study. All the mothers had lived in Uganda for the past 5 years and only one had traveled to Kenya.

RTI resistance-associated mutations

None of the 37 strains under investigation showed any major mutation that is known to be associated with drug resistance to any RTI class. Therefore, according to the WHO HIVDR Threshold Surveillance protocol our study population falls below the threshold of 5%, which is used as a marker to define well-functioning ART programs.

Protease sequence variability in isolates from newly diagnosed non-B-infected individuals

The amino acid sequence of each strain was compared to the subtype B consensus amino acid sequence using the HIV drug resistance published algorithm from the IAS-USA[15] for

Table 2. Distribution of Natural Polymorphisms in the Protease Sequences of Newly Diagnosed HIV-1-Infected Ugandans[a]

Subtype	n *(%)*	*L10I/V*	*I13V*	*K20R*	*M36I*	*L63P*	*H69K*	*A71V/I/T/L*	*V77I*
A1	22 (48.0)	3	19	4	22	9	19	0	0
A2	1 (2.0)	1	0	0	1	0	0	0	0
C	1 (2.0)	0	0	0	1	0	1	0	0
D	18 (39.0)	1	11	5	12	5	0	1	1
URFs	4 (9.0)	1	2	0	4	1	1	0	0
	46 (100)	6 (13.0)	32 (69.5)	9 (19.5)	40 (87.0)	15 (32.6)	21 (46.0)	1 (2.0)	1 (2.0)

[a]The frequencies of the different mutations according to subtype. The letter represents the amino acid substitution and the number indicates the number of strains with this mutation.

mutations associated with resistance to PIs and RTIs. In contrast, many secondary mutations and naturally occurring polymorphisms were found at the following positions, in order of decreasing frequency: M36I [$n = 40$ (87%)], I13V [$n = 32$ (69.5%)], H69K [$n = 21$ (46%)], L63P [$n = 16$ (33%)], K20R [$n = 9$ (19.5%)], L10I/V [$n = 6$ (13%)], A71V [$n = 1$ (2%)], and A77I [$n = 1$ (2%)]. No major mutations (D30N, V32I, M46I, I47V/A, G48V, I50L, I54M/L, L76V, V82A/F/T, I84V, N88S, or L90M) were seen in any of the non-B strains from our samples. Table 2 summarizes the frequencies of the different mutations according to subtype. A71V and A77I were subtype D-specific mutations and thus were not statistically significant.

A separate "sampling and classification plan" (data not shown) was filled in for each ARV drug or drug class of interest.

Discussion

This study provides the most recent data on the molecular characterization of HIV-1 in Entebbe, South Central Uganda. At least three genetic subtypes [A (sub-subtype A1 and A2), C and D] and four unique recombinant forms (A1/D) have been identified in recently HIV-1-infected pregnant adolescents and young pregnant females attending an antenatal clinic in Entebbe. The subtype distribution characteristics described for this small dataset are consistent (dominated by subtype A and D, with a small number of subtype C infections) with those previously published in Uganda.[9,18,19]

We further carried out a Wilcoxon rank-sum test (Mann–Whitney two-sample statistic) on specimens with CD4 count ≥ 350 and < 500 cells/mm^3 to determine which subtype was more associated with low CD4 cell counts. Results indicated that of the nine samples, three belonged to subtype A1 (33.3%), five (56.6%) to subtype D, and one (1.1%) intersubtype recombinant with a p value of 0.09. With some caution we can speculate on the relationship between CD4 count and the subtype. We have previously shown that there seems to be an earlier switch from R5 to X4 viruses among subjects infected with subtype D compared to subtype A, and this may be one of the reasons for differences in pathogenicity between these subtypes.[20]

None of the specimens with one or more known mutations associated with HIVDR has been found after the 37th specimen was genotyped. This is similar to previous findings in several countries that have implemented the WHO threshold surveillance approach.[21–24] Analysis of amino acid variation in this dataset at resistance positions revealed that while variation was minimal between subtypes A and D in RT, there is appreciable variation in PR at minor positions. While in isolation these changes do not have a direct impact on PI resistance per se, there is some evidence that these minor mutations are similarly compensatory for many PIs. As a result, the preexistence of minor compensatory mutations in subtype A and D might result in the faster emergence of viruses resistant to PIs. Until the spectrum of accessory mutations for subtype A and D viruses is more fully characterized, this cannot be confirmed.[9,18] Preliminary data suggest that prior M36I and L10I/V mutations are associated with a more rapid fall in sensitivity during treatment.[25]

An obvious challenge in resource-limited settings such as Uganda will be a balance between rapid introduction of ART and continual surveillance of drug resistance to prevent treatment failures and to avoid a public health crisis.[12] A national HIV drug resistance prevention, surveillance, and monitoring plan based on the WHO HIVDR prevention strategies is being implemented to address this issue with a WHO accredited National HIV drug resistance laboratory at the Uganda Virus Research Institute, Entebbe.

Acknowledgments

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